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ORIGINAL PAPER

An Efficient Binary Solvent Mixture for Monoacylglycerol Synthesis by Enzymatic Glycerolysis

Nanjing Zhong · Lin Li · Xuebing Xu · Lingzhi Cheong · Bing Li · Songqing Hu · Xihong Zhao

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Abstract The present study was aimed at selecting an efficient binary solvent mixture for monoacylglycerol (MAG) synthesis by enzymatic glycerolysis of soybean oil. Solvent combinations of *tert*-butanol/isopropanol (v/v) at different ratios were studied. Of the investigated cases, *tert*-butanol:isopropanol at ratio 80:20 was the most suitable organic medium. The optimum conditions for MAG synthesis under the selected mixture were: water 10 wt% based on glycerol, Lipozyme TL IM 15 wt% based on oil and glycerol, weight ratio of solvent to oil 4:1, and molar ratio of glycerol to oil 3.5:1. Under these conditions with a 4-h reaction, the yield of MAG was 72.0% where the triacylglycerol (TAG) content was reduced to only 1.0% (based on acylglycerols). Fatty acid ester (FAE) formation from the solvents was very low in the final product (1.3% based on reaction mixture). The selected binary solvent mixture has good physical properties with low melting point (−26.5 °C), which can avoid the risk of crystallization in practical operations.

Keywords Glycerolysis · Monoacylglycerol · Lipozyme TL IM · Binary solvent medium · Soybean oil

N. Zhong · L. Li · B. Li (✉) · S. Hu · X. Zhao
College of Light Industry and Food Sciences,
South China University of Technology,
510640 Guangzhou, People's Republic of China
e-mail: lcbingli@scut.edu.cn

N. Zhong
e-mail: adong473@163.com

X. Xu · L. Cheong
Department of Molecular Biology,
Aarhus University, Gustav Wieds Vej 10,
8000 Aarhus C, Denmark

Introduction

Monoacylglycerols (MAG) are recognized as safe food-grade additives by the American Food and Drug Administration and the EU. MAG, or their mixtures with diacylglycerols (DAG), account for approximately 75% of worldwide emulsifier production, which has been estimated at about 200,000–250,000 metric tons per year. MAG are widely used as emulsifiers in the food, pharmaceutical and cosmetic industries. They are also used as synthetic intermediates and as chiral building blocks in synthetic organic chemistry [1–5].

Conventionally, commercial MAG are widely manufactured by chemical glycerolysis of oils and fats at high temperatures of 200–250 °C, using inorganic alkaline such as NaOH or $Ca(OH)_2$ as catalysts [3, 4, 6]. The process presents some drawbacks such as dark color, burnt taste, and high energy consumption. In particular, it is not suitable for heat-sensitive MAG production [1, 3, 4].

MAG can be produced through direct esterification of glycerol with fatty acids (FA). Macrae et al. [7] reported a reaction product containing >95 mol% MAG by using potato lipid acyl hydrolase to synthesize MAG from glycerol and FA. Since then, the use of enzymes as catalysts has received considerable attention for several years because of the much lower temperature required [2–6, 8]. However, at low temperatures, glycerol and the oil are immiscible, hence enzymatic glycerolysis of tricaylglycerols (TAG) typically yields only 30–50% of MAG [3, 5]. To enhance the reaction efficiency, various strategies have been reported [9–11]. A two-step temperature programming method was found to be helpful for higher MAG yield in which the reaction mixture was first incubated at 42 °C for 8–16 h followed by reducing the reaction temperature to 5 °C for up to 4 days. The protocol is limited as

the long reaction time caused difficulties in reusability of enzymes. In addition, low reaction temperature also led to high viscosity of the reaction mixture which subsequently decreased mass transfer and the overall reaction rate.

The introduction of organic solvents to improve the solubility of the reactants seems to be a potential alternative and has been investigated extensively in recent years. Pure solvents, like *tert*-butyl methyl ether (MTBE), *tert*-butanol (TB) and *tert*-pentanol (TP), or binary mixtures of *n*-hexane with TB or TP, are quite beneficial for lipase-catalyzed MAG synthesis [1–4, 8, 9, 12]. Damstrup et al. [4] found a binary mixture of TB:TP (80:20 vol%) to be efficient in producing high MAG yield (47–56%) in a short time (20 min). Some other solvents like isooctane, acetone, or binary mixtures of acetone and isooctane have been shown to be useful in lipase-catalyzed glycerolysis reactions [5, 8, 13].

From an industrial point of view, miscibility and operation feasibility are some of the parameters to be considered in selecting appropriate solvent systems for lipase-catalyzed glycerolysis. Due to its high melting point (25 °C), TB has a risk of crystallization and thus making solvent recovery difficult. Therefore, partial substitution of TB with low melting solvents would increase the feasibility of industrial MAG synthesis through lipase-catalyzed glycerolysis.

In the present study, isopropanol (IP) was studied for partial substitution of TB in MAG synthesis through lipase-catalyzed glycerolysis. Instead of Novozym 435 (*Candida antarctica* Lipase B) which was commonly used in enzymatic glycerolysis, this study used the considerably cheaper Lipozyme TL IM. The reaction conditions of water content, enzyme concentration, substrate molar ratio, reaction time, solvent combination and dosage were evaluated. The formation of by-products, namely free fatty acids (FFA) and fatty acid alcohol esters (FAE), was examined. The physical behavior of the selected binary mixture, i.e., its melting point, was also studied to ensure a proper operational feasibility.

Materials and Methods

Materials

The *sn*-1, 3 specific Lipozyme TL IM (immobilized *Thermomyces lanuginosus* lipase) was obtained from Novozymes (Beijing, China) and soybean oil was kindly provided by Kerry Oleochemical Industrial (Shanghai, China). The FA composition (% w/w) of the soybean oil was: C16:0, 12.1; C18:0, 4.7; C18:1, 24.8; C18:2, 50.7; C18:3, 6.9; C20:0, 0.4; C22:0, 0.4. Glycerol with a purity of more than 99.0% was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). The standards of glycerol (>99.5%), 1-monoolein, 1,3-diolein and triolein (>99.0%) for HPLC analysis were from Sigma (St. Louis, MO). Fatty acid methyl ester (FAME) standards for GC analysis were purchased from Fluka (Buchs, Switzerland). All other solvents and reagents were of analytical or chromatographic grades.

Enzymatic Glycerolysis of Soybean Oil

Enzymatic glycerolysis of soybean oil was conducted in a 50-mL capped flask under a fixed stirring rate of 520 rpm and reaction temperature of 45 °C. The reaction conditions of water content, enzyme concentration, substrate molar ratio, reaction time, solvent combination and dosage were studied at the following levels: water content (2–12 wt% of glycerol), enzyme concentration (5–20 wt% of oil and glycerol), substrate molar ratio [glycerol:oil = 2.5:1-6.5:1 (mol:mol)], reaction time (1–8 h), solvent combination [TB:IP = 1:0-0:1 (wt:wt)], and solvent dosage [solvent:oil = 0.1:1-5:1 (wt:wt)]. Firstly, 8.8 g soybean oil and desired amount of glycerol, water and solvent were mixed by stirring. Reaction was initiated by addition of the lipase. At the end of the reaction, 1 mL of the reaction mixture was withdrawn and dissolved in 10 mL of chloroform. The mixture was then filtered through a microfilter (0.45 μm) to remove the lipase. All samples were stored at −20 °C prior to analysis.

Determination of MAG, DAG and TAG by HPLC

The lipid profile was analyzed by a reversed-phase high-performance liquid chromatography (HPLC). The chromatographic apparatus consisted of a P680HPLC Pump with a quaternary gradient system, an ASI-100 Automated Sample Injector, a Thermostatted Column Compartment TCL-100 (all from DIONEX, Sunnyvale, CA, USA), and an ELSD detector ELSD2000ES (Alltech, USA).

The chromatographic separation of the compounds was carried out with a Diamonsil C18 column (250 × 4.6 mm i.d., particle size 5 μm) and the column temperature was hold constant at 40 °C. Gradient elution was achieved by mobile phases A (acetonitrile:acetic acid = 99.95:0.05, v/v), B (dichloromethane), C (water), by volume. The course of the gradient was as followed: 0–2 min: 80% A and 20% C; 2–12 min: change to 100% A; 12–20 min: change to 70% A and 30% B; 20–26 min: maintain 70% A and 30% B; 26–35 min: change to 30% A and 70% B; 35–37 min: maintain 30% A and 70% B; 37–39 min: change to 100% A; 39–42 min: maintain 100% A. The injection volumes of 10 μL and the elution flow-rate of 1 L/min were used in all

experiments. The effluent was monitored by an ELSD detector with evaporator temperature at 75 °C and the flow-rate of the nitrogen at 1.7 L/min. The results were based on double measurements (Fig. 1).

Compounds were identified by HPLC/MS. The HPLC conditions were the same as described above. An Esquire HCT Plus analyzer (Bruker Daltonics, Bremen, Germany) was equipped with a positive-ion atmospheric pressure chemical ionization (APCI) source and a positive-ion electrospray ionization (ESI) source. To obtain better results, ESI was taken for MAG identification and APCI for DAG and TAG. The following tuning parameters were used. APCI: the pressure of the nebulizing gas was 25 psi, the drying gas flow rate was set at 5 L/min, the temperatures of the drying gas and APCI heater were 250 and 350 °C, respectively. ESI: the pressure of the nebulizing gas was 20 psi, the drying gas flow rate was set at 12 L/min, the temperature of the drying gas was 300 °C.

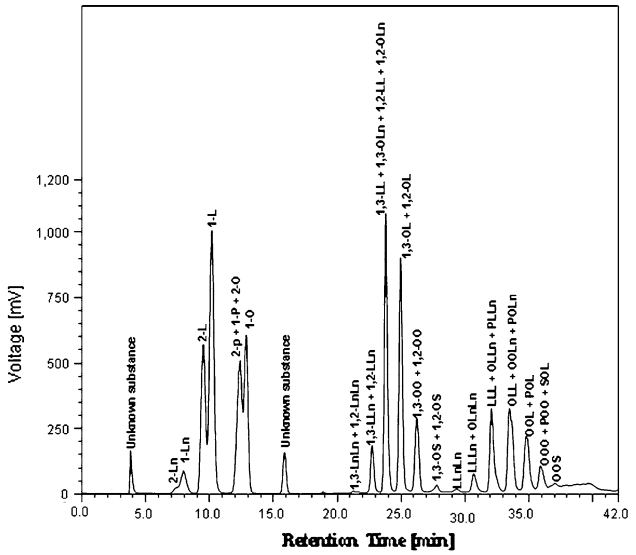

Fig. 1 Chromatographic separation of MAG, DAG, and TAG by means of HPLC. For detailed description, see "Methods". Notation of compounds *2-Ln* 2-monolinolenin, *1-Ln* 1-monolinolenin, *2-L* 2-monolinolein, *1-L* 1-monolinolein, *2-P* 2-monopalmitolein, *1-P* 1-monopalmitolein, *2-O* 2-monoolein, *1-O* 1-monoolein, *1,3-LnLn* 1,3-dilinolenin, *1,2-LnLn* 1,2-dilinolenin, *1,3-LLn* 1,3-linoleoyl-linolenoyl-glycerol, *1,2-LLn* 1,2-linoleoyl-linolenoyl-glycerol, *1,3-LL* 1,3-dilinolein, *1,3-OLn* 1,3-oleoyl-linolenoyl-glycerol, *1,2-LL* 1,2-dilinolein, *1,2-OLn* 1,2-oleoyl-linolenoyl-glycerol, *1,3-OL* 1,3-oleoyl-linoleoyl-glycerol, *1,2-OL* 1,2-oleoyl-linoleoyl-glycerol, *1,3-OO* 1,3-diolein, *1,2-OO* 1,2-diolein, *1,3-SO* 1,3-stearoyl-oleoyl-glycerol, *1,2-SO* 1,2-stearoyl-oleoyl-glycerol, *LLnLn* dilinolenoyl-linoleoyl-glycerol, *LLLn* dilinoleoyl-linolenoyl-glycerol, *LLL* trilinolein, *OLnLn* dilinolenoyl-oleoyl-glycerol, *OLLn* oleoyl-linoleoyl-linolenoyl-glycerol, *PLLn* palmitoyl-linoleoyl-linolenoyl-glycerol, *OLL* dilinoleoyl-oleoyl-glycerol, *OOLn* dioleoyl-linoleoyl-glycerol, *POLn* palmitoyl-oleoyl-linolenoyl-glycerol, *OOL* dioleoyl-linoleoyl-glycerol, *POL* palmitoyl-oleoyl-linoleoyl-glycerol, *OOO* triolein, *POO* palmitoyl-dioleoyl-glycerol, *SOL* stearoyl-oleoyl-linoleoyl-glycerol, *OOS* dioleoyl-Stearoyl-glycerol

Relative quantification was used under the assumption that each individual component categories had same responses. Thus, the relative quantification calculation taking MAG content (based on MAG, DAG, and TAG), for example, was as follows:

$$\mathrm{MAG}\% = \frac{A_M \times \frac{1}{S_M}}{A_M \times \frac{1}{S_M} + A_D \times \frac{1}{S_D} + A_T \times \frac{1}{S_T}} \times 100$$

where A_M, A_D and A_T is the sum of peak areas of MAG, DAG, and TAG, respectively. S_D is the relative response factor value of 1,3-diolein, S_T is the relative response factor value of triolein. Response factor values were given in relation to 1-monoolein, and thus $S_M = 1$.

By-Product Analysis

FFA content was determined by KOH titration according to the standard method [14]. Meanwhile, FAE content in the product, due to the reactions with alcohol solvents, was determined by a Hewlett-Packard Model 6890 gas chromatograph (GC) (Palo Alto, CA) equipped with a FID and a DB-FFAP methyl-silicone capillary column (15 m × 0.32 mm i.d., film thickness 0.1 μm; J&W Scientific, Folsom, CA). The samples were dissolved in chloroform with hendecanoic acid ester (2 mg/mL) as an internal standard. The oven temperature was programmed from initial 150, after keeping for 2 min, to 230 °C at a rate of 10 °C/min, and maintained the temperature for 8 min. The injector and FID detector temperatures were 250 and 300 °C, respectively. The carrier gas was nitrogen at a flow rate of 25 mL/min. The fatty acid isopropyl esters or *tert*-butyl esters were identified by comparing their retention time with their corresponding fatty acid methyl esters, for example, palmitic acid isopropyl ester or *tert*-butyl ester corresponding with the standard palmitic acid methyl ester. The results were calculated based on response factors from the standard.

Melting Point Measured by Differential Scanning Calorimetry (DSC)

The transformation from solid to liquid of the selected binary mixtures was investigated by DSC (DSC Q20; TA Instruments, New Castle, DE). The weighed sample was hermetically sealed in an aluminum plate with an empty plate serving as a reference. The sample was analyzed by initially cooling from 20 to −60 °C at 10 °C/min and held at −60 °C for 5 min. Subsequently, the sample was heated from −60 to 50 °C at a rate of 5 °C/min. The result was based on double determinations.

Results and Discussion

Effect of Water Content

For most lipases, water is necessary to maintain the catalytically-active conformation and to allow the formation of an acyl-enzyme complex [3], while some other lipases such as Novozym 435 (a commercially available preparation of immobilized *Candida antarctica* lipase B, CALB) can remain highly active in a dry state without any water addition [15]. Thus, it is important to determine the optimal water content in the reaction mixture as it not only affects lipase activity but also the reaction rate and product yield. For example, Yamane et al. [16] found that too high water content did not enhance the MAG yield; instead, it increased the by-product formation, such as FFA. Figure 2 shows the effect of water content on MAG yield from lipase-catalyzed glycerolysis of soybean oil. MAG yield increased with the increase of water content. The highest yield of MAG was obtained when 10 wt% water was added. Further increase in water content beyond 10 wt% led to a reduction in MAG yield which was probably caused by hydrolysis due to the high content of water. In addition, it might be due to the limitation of substrate transport from the reaction medium to the vicinity of the enzyme at high water content. Instead of forming a thin mono hydration layer for catalytically-active conformation, excess water surrounded the particle of the biocatalyst preventing lipophilic substrate access to the enzyme and so led to the particle aggregation [15, 17].

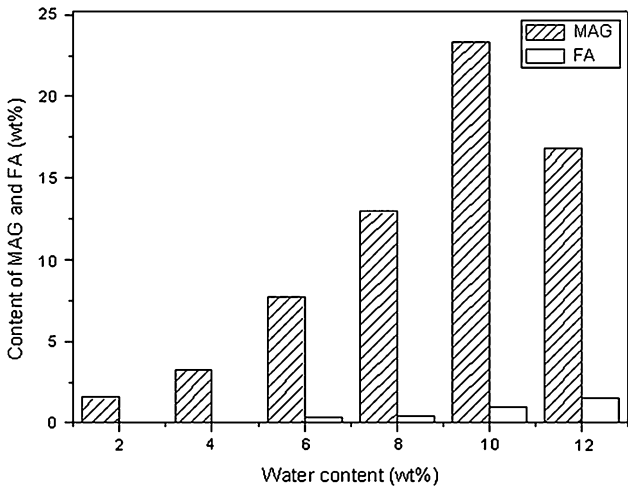

Fig. 2 Effect of water content on glycerolysis of soybean oil. Reaction conditions: glycerol/oil 4.5:1 (mol/mol), 15 wt% TL IM (based on oil and glycerol), TB/IP 80:20, solvent mixtures (TB/IP 80:20)/oil 2:1 (wt/wt). The reaction was carried out at 520 rpm and 45 °C for 8 h

Effect of Enzyme Concentration

At certain ranges, there is a linear relationship between the enzyme concentration and the reaction rate. Increase in enzyme concentration leads to increase of reaction rate. The effect of Lipozyme TL IM concentration on MAG production was investigated. The results are shown in Fig. 3. On increasing the amount of Lipozyme TL IM in the reaction mixture, the MAG formation increased. However, no further increment was obtained beyond 15 wt% lipase. It may be due to the fact that the active sites of the enzyme molecules present in large excess would not be exposed to the substrates which are possibly caused by protein aggregation [18]. The present results agreed with a previous study which showed a similar trend of MAG production upon increasing the enzyme amount [3]. Therefore, Lipozyme TL IM at 15 wt% was used for further study.

Effect of Solvent Dosage

The solvent plays multiple roles in the reaction system. It can help to improve the system homogeneity and stability as well as increase the mass transfer by reducing the viscosity of the system. Nevertheless, polar solvents, such as IP and ethanol, may strip off essential water from the lipase structure [3, 19]. In this situation, more water should be added or the lipase activity would be decreased. Furthermore, solvent may reduce the concentration of substrates, which in turn influences the reaction rate according to the Michaelis–Menten equation [20]. Therefore, it is important to determine optimal solvent dosage. The effect of solvent dosage on MAG production is shown in Fig. 4. A sharp

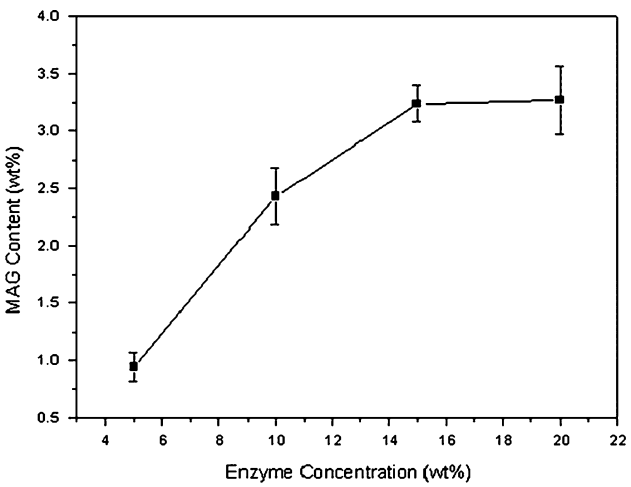

Fig. 3 Effect of enzyme concentration on MAG production. Reaction conditions: glycerol/oil 4.5:1 (mol/mol), TB/IP 80:20, solvent mixtures (TB/IP 80:20)/oil 2:1 (wt/wt), water added 4 wt% based on glycerol. The reaction was carried out at 520 rpm and 45 °C for 8 h

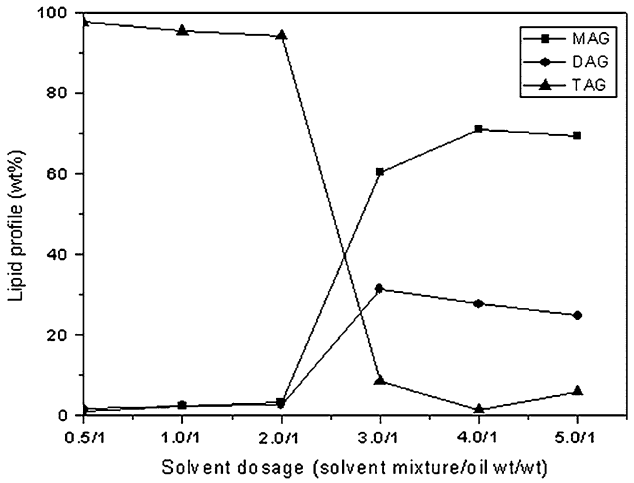

Fig. 4 Effect of solvent dosage on glycerolysis of soybean oil. Reaction conditions: glycerol/oil 4.5:1 (mol/mol), TB/IP 80:20, water added 4 wt% based on glycerol, 15 wt% TL IM (based on oil and glycerol). The reaction was carried out at 520 rpm and 45 °C for 8 h

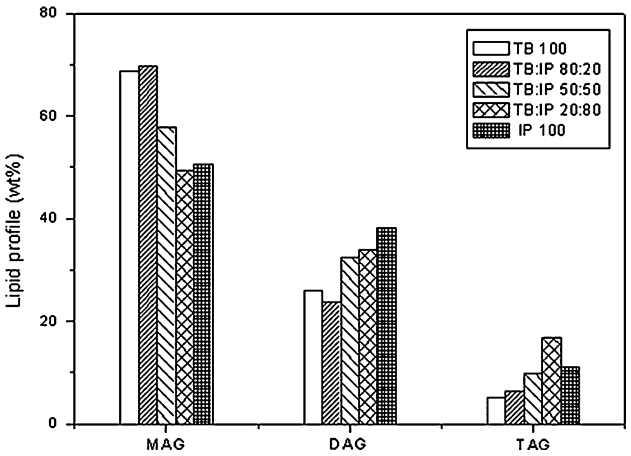

Fig. 5 Effect of solvent combination on glycerolysis of soybean oil. Reaction conditions: glycerol/oil 4.5:1 (mol/mol), water added 4 wt% based on glycerol, 15 wt% TL IM (based on oil and glycerol), solvent mixtures (TB/IP 80:20)/oil 4:1 (wt/wt). The reaction was carried out at 520 rpm and 45 °C for 8 h

increase in MAG yield was observed when the solvent dosage was increased from 2:1 to 3:1 (solvent:oil in wt ratio). There was no further increment beyond 4:1. Yang et al. [20] showed that TB to sunflower oil in the weight ratio 1.5:1 was the best for a batch reaction system to produce MAG containing polyunsaturated fatty acids. In the present study, the ratio 4:1 was selected for the glycerolysis reaction.

Effect of Solvent Combination

The effect of solvent combination (TB/IP, v/v) at different ratios on MAG formation is demonstrated in Fig. 5. Of the five different solvent mixtures, the highest MAG yield was achieved by reacting at 80:20 vol% TB:IP system. IP is more hydrophilic than TB due to one less carbon atom placed in the alcohol chain. Addition of IP to TB leads to the increase of solvent polarity which helps to enhance substrate access to the active site of the enzyme and thus leads to the higher MAG formation. Nevertheless, further increment of IP beyond 20% led to a reduction in MAG yield. This may be due to inactivation of biocatalyst caused by the more polar solvent. A previous report has shown that solvent properties influenced the activity and stability of the enzyme to a large extent [19]. Usually, hydrophobic solvents cause less inactivation than more hydrophilic solvents.

In fact, Damstrup et al. [2] had tried to correlate log *P* values and MAG contents by evaluating 13 different solvent systems with a wide range of log *P* values; log *P* is defined as the logarithm of the partition coefficient of a substrate in the standard 1-octanol–water two-phase system. Their results showed that the maximum MAG contents were achieved with solvents having a tertiary alcohol structure while the correlation with log *P* values was not completely followed even though for the suitable solvents with log *P* values in the range of 0.3–1. In any case, the TB:IP 80:20 vol% solvent system was selected for further investigation in the present study.

Effect of Substrate Molar Ratio

Substrate molar ratio has different effects on lipase-catalyzed glycerolysis. The increase of glycerol amount will increase the theoretical equilibration values which increase the yield of MAG accordingly. Furthermore, glycerol can act as an effective stabilizer against thermal and solvent deactivation [5, 21–23]. Nevertheless, glycerol content in the substrate will also influence the system polarity, which subsequently affects the system stability and homogeneity [20]. In addition, especially for enzymes with a hydrophilic carrier, glycerol which is hydrophilic may form a coating surrounding the enzyme which subsequently led to enzyme clumping and inactivation [17].

The effects of glycerol/oil molar ratio are given in Fig. 6. From the thermodynamic point of view, at certain ranges, more glycerol would produce more MAG, as shown in the figure. However, there is little difference in MAG yield between the 3.5:1 and 4.5:1 glycerol/oil molar ratios. This may possibly be due to the above-mentioned effect of glycerol on the system homogeneity. On the other hand, the results for ratios 5.5:1 and 6.5:1 were a bit confusing. The above considerations cannot be fully explained, and a special phase study might be needed to look into the phenomenon. For DAG and TAG, DAG content dropped gradually as the glycerol/oil molar ratio increased in the

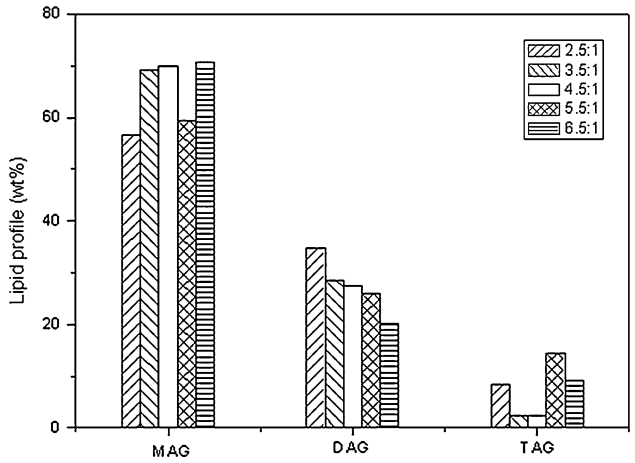

Fig. 6 Effect of glycerol/oil molar ratio on glycerolysis of soybean oil. Reaction conditions: water added 4 wt% based on glycerol, 15 wt% TL IM (based on oil and glycerol), solvent mixtures (TB/IP 80:20)/oil 4:1 (wt/wt). The reaction was carried out at 520 rpm and 45 °C for 8 h

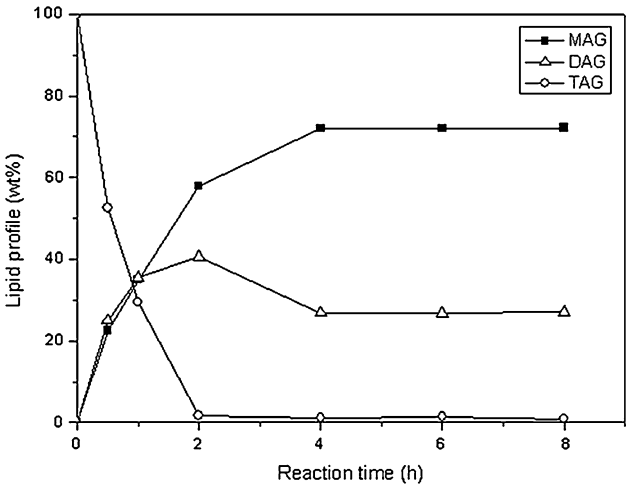

Fig. 7 Glycerolysis time courses of soybean oil. Reaction conditions: water added 10 wt% based on glycerol, 15 wt% TL IM (based on oil and glycerol), solvent mixtures (TB/IP 80:20)/oil 4:1 (wt/wt), glycerol/oil 3.5:1 (mol/mol). The reaction was carried out at 520 rpm and 45 °C

studied range, while TAG yield was the lowest in the molar ratio of 3.5:1 and 4.5:1, and the highest in 5.5:1. Glycerol/oil molar ratio at 3.5:1 was selected for the present study. This result is a bit different from previous studies. Pawongrat et al. [3] used MTBE as solvent and showed that the optimum molar ratio of glycerol to tuna oil for MAG production was 3:1, while Yang et al. [20] showed the molar ratio of glycerol to sunflower oil at 4.5:1 was the best for the production of MAG containing polyunsaturated fatty acids, in which TB was employed as the solvent system.

MAG Production under Optimal Conditions

The optimal conditions for MAG production were as follows: water added based on glycerol 10 wt%, the amount of Lipozyme TL IM based on oil and glycerol 15 wt%, the weight ratio of solvent to oil 4:1, solvent combination (TB/IP, v/v) 80/20, and molar ratio of glycerol to oil 3.5:1. Temperature was controlled at 45 °C. The results are shown in Fig. 7. Under the optimized conditions, with a 4-h reaction, the yield of MAG was 72.0%, while the TAG content was reduced to only 1.0%.

FAE Content of the Product

Primary and secondary alcohols can participate in lipase-catalyzed esterification reactions with FA [2, 24]. The formation of unwanted FAE in the glycerolysis reaction in binary solvent combination (TB/IP, v/v) systems at different ratios was determined. FAE content based on oil and glycerol increased according to the increase of the IP volume ratio in the solvent mixture (data not shown). The results corresponded to the fact that secondary alcohols can react with FA more easily than tertiary alcohols based on the consideration of steric structures. The FAE content was only marginal (1.3%) in the selected TB:IP 80:20 vol/vol solvent system, while no FAE can be detected in the pure TB system in the present study. Interestingly, in Damstrup et al.'s study [2], FAE content was 1.9 and 0.6% after reaction in the TP and TB systems, respectively.

Melting Point of the Selected Binary Mixture

As mentioned before, pure TB solvent has a risk of crystallization during the purification process after glycerolysis reaction, while partial substitution of TB is a potential as well as imperative alternative for practical considerations [4]. Since the boiling point of TB and IP is almost the same, both at 82 °C [2], theoretically, the boiling point of the TB/IP mixture would be similar. The melting point of the selected mixture was determined using DSC and the results are shown in Fig. 8. Replacement of TB with IP greatly lowered the melting point (to −26.5 °C). This is sufficient to avoid practical problems with crystallization based on the report from Damstrup et al. [4], who pointed out that solvent with a melting point below 15 °C and a temperature range between the melting and boiling points of at least 65 °C was considered to be sufficient for practical operations.

Conclusions

The selected solvent combinations resulted in high yield of MAG, up to 72.0% while with a low FAE content of 1.3%.

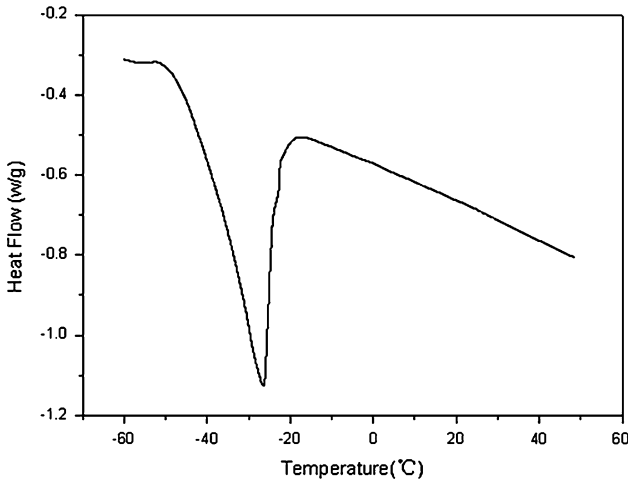

Fig. 8 DSC profile of TB/IP = 4/1 (v/v) mixture. The sample was analyzed by initially cooling from 20 to −60 °C at a rate of 10 °C/min and held at −60 °C for 5 min. Subsequently, the sample was heated from −60 to 50 °C at a rate of 5 °C/min

The melting point of the selected solvent mixture is −26.5 °C, which can avoid the risk of crystallization in practical operations. Thus, it could have potential for industrial considerations.

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